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16. ABSTRACT

Butyl benzyl phthalate (BBP) is a commerically important phthalate ester identified as a priority pollutant by the Environmental Protection Agency. The Clean Water Act of 1977 (PL 95-217) requires the Administrator of the EPA to publish criteria for water quality that accurately reflect current scientific knowledge of the extent and effects of pollutants present in water bodies. Section 304(a)(1) of the Act provides for the nonregulatory, scientific assessment of ecological effects. This work was conducted to provide data for Section 304 on the toxicity of BBP to saltwater fish. The 96-h LC_{50} values found for English sole, *Parophrys vetulus*, were 0.66 (static replenish exposure) and 0.55 mg/L (flow-through exposure) with lethal threshold concentrations of 0.45 and 0.30 mg/L, respectively. Sublethal effects were noted at all BBP exposures, the lowest of which was 0.1 ppm.

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Acute Toxicity of Butylbenzyl Phthalate to Shiner Perch (*Cymatogaster aggregata*)

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Abstract. The Clean Water Act of 1971 directed the Environmental Protection Agency to establish ambient water quality criteria for several classes of elements and compounds, including phthalate esters. Multispecies acute toxicity data are required to develop these criteria. Static bioassay LC_{50}s for butylbenzyl phthalate (BBP) for a single species of marine fish based on nominal doses range from 3 mg/L to 440 mg/L. Flow-through bioassays were used in this study of BBP with shiner perch (*Cymatogaster aggregata*). Using measured exposure concentrations, 96-hr LC_{50}s averaged 0.51 mg/L. Effects on schooling behavior were found at 0.08 mg/L and coloration at 0.24 mg/L. Coupled with the behavioral changes, reduced brain levels of epinephrine found in surviving fish indicated that the mode of acute toxicity for BBP may be through its effects on the catecholamines of the central adrenergic nervous system.

Butylbenzyl phthalate (BBP) is a member of a class of compounds which are esters of phthalic anhydride. These esters are used extensively in the plastics industry to make polymerized materials such as polyvinyl chloride more flexible. Approximately 9.5% of the 1980 United States phthalate ester production (479×10^6 kg) was BBP (Gledhill *et al.* 1980; U.S. International Trade Commission 1980).

BBP causes testicular atrophy in rats (Gray and Butterworth 1980). However, data on teratogenicity and carcinogenicity for this compound have been equivocal (USEPA 1980b; U.S. Department of Health and Human Sciences 1981). BBP and other phthalate esters have been consistently found in the surface waters (USEPA 1977; Sheldon and Hites 1978) and freshwater sediments of the United States (Gledhill *et al.* 1980). These compounds are present in the liquid and solid fractions of many sewage treatment plant effluents (USEPA 1980a) which in the coastal areas are often discharged into the marine environment where high concentrations have been found in the sediments near outfall pipes.

As a result of the ubiquity of BBP in environmental samples and its high production, the U.S. Environmental Protection Agency (USEPA) included it with several other phthalate esters in a list of toxic pollutants (USEPA 1978) pursuant to Section 307(a)(1) of the Clean Water Act of 1977. The EPA is required by Section 304(a)(1) of the Act to publish criteria for marine water quality results of acute bioassays for at least two different fish families (USEPA 1980c).

Using static bioassays with nominal BBP concentrations, Heitmuller *et al.*(1981) and Gledhill *et al.* (1980) found 96-hr LC_{50} values of 440 mg/L and 3 mg/L, respectively, for BBP with the sheepshead minnow (*Cyprinodon variegatus*), a marine fish. The work presented here was conducted to add toxicity data for shiner perch (*Cymatogaster aggregata*) and to relate toxicity to measured rather than nominal doses. Tissue chemistry and behavior observations were done to aid in evaluating environmental exposure levels.

Materials and Methods

Holding and Acclimation Procedures

Juvenile shiner perch (euryhaline, estuarine species) were collected by beach seine from Yaquina Bay, Oregon during the

summer of 1981. ASTM Standard Practices (1980) were used for guidance. The fish were initially quarantined for one week. Healthy fish were transferred to holding tanks for four weeks followed by a transfer to an acclimation room for at least one week prior to experimentation. All tanks were made with silicone adhesive (RTV 108, General Electric Company)[1] and 6-mm thick glass. Each 270-L tank was 50.8 cm high by 91.4 cm by 61.0 cm. The fish were exposed to water drawn from Yaquina Bay at a depth of 3 m below mean sea level. Raw seawater was used during quarantine and holding, while sand-filtered and UV-sterilized water was used during acclimation. Dissolved oxygen concentrations were maintained at greater than 80% of saturation by aeration, and replacement water flow rates exceeding two volumes per day. The biomass loading was less than 2 g of fish per L (fewer than 150 fish per tank). The quarantine/holding room was sunlighted while the acclimation room was on an 16/8hr light/dark cycle with no transition. The salinity of the incoming seawater was between 30‰ and 34‰ throughout the holding and bioassay periods. A ration of Oregon Moist Pellets (Crawford and Law 1972) was given at a rate of approximately 4% (wet weight of food per wet weight of fish) per working day.

Bioassay Procedures

A flow-through exposure system was used for the bioassays. Gravity flow of six dilution water streams from a headtank was controlled by screw pinchcocks to obtain a 10-volume per day flow rate through each treatment chamber. Solutions of BBP in 95% ethanol were pumped into each of five dilution water streams at nominal flow rates of 0.05 ml/min using solvent flexible tubing in a Technicon AutoAnalyzer pump. A sixth tube was used to pump only 95% ethanol into the control dilution water stream. Standard pump tubing was unacceptable because it rapidly deteriorated while pumping 95% ethanol. This deterioration was apparently caused by the alcohol leaching a soluble component of the tubing. Concentrations as high as 288 mg/L of the common plasticizer (2-ethylhexyl) phthalate were found in 95% ethanol pumped through this type of tubing. The 12°C ± 1°C bioassay temperature was maintained by controlling the air temperature of the bioassay room and passing the sand-filtered and UV-sterilized dilution water through a large-volume stainless steel chiller. The treatment tanks were holding tanks partitioned lengthwise into three 20-L chambers (31.7 cm high by 68.0 cm by 20.0 cm) with 3-mm glass walls.

The wall opposite the inflow was perforated at the bottom and an outer wall controlled the depth of water within the treatment chambers to 15.2 cm as water passed under the inner wall and flowed over the outer wall to drain. This resulted in a volume of approximately 20 L per chamber.

Range-finder bioassays were used to select the five BBP concentrations of each bioassay. A sixth tank containing seawater with the ethanol carrier solvent served as the control treatment for the bioassays. A seawater-only control was not used as no mortalities in any of our ethanol controls have been observed using this species or English sole (*Parophrys vetulus*) (Randall *et al.* (in press). The ethanol concentration never exceeded 0.5 ml/L.

A bioassay was started by placing 20 fish (3 to 5 at a time) into filled and flowing, randomly chosen chambers. The fish averaged 3.0 g ± 1.0 g ($\bar{x} \pm s$, $n = 40$). Observations on water quality, fish behavior, and mortality were taken at the frequency recommended by ASTM (1980). The water quality values were found to be within the recommended ranges for flow-through bioassays. Salinities for all bioassays were from 31 ‰ to 32 ‰. Behavioral observations included: the presence or absence of a coughing response (Belding 1929); activity level as manifest in the swimming and turning rate; and school density as compared to the control fish which occupied one-half to one-third of the chamber at the inflow end. The darkness of the dorsal side of the fish was also noted.

Analysis of Butylbenzyl Phthalate

The sample extraction and concentration procedure was a modification of a methodology described by Andrews and Good (1982) for the extraction of pesticides from water. All glassware was combusted at 300°C for eight hr.

Samples were taken from the middle of a chamber at mid-depth using a glass siphon with a pinch-clamped latex rubber tube at the end. Several siphon volumes were allowed to pass through before a 100 ml sample was drained into a calibrated glass bottle (Wheaton). The contents were shaken with 10 ml of added glass-distilled methanol and then drawn through a conditioned 200 mg, C-2, Bond Elut® column (Analytichem International, Harbor City, CA) in a Vac Elut® manifold at a vacuum of 380 mm of Hg. Following two rinses with 2 ml of double glass-distilled water, the retained BBP was eluted from the column with 2 ml of double distilled acetonitrile at a vacuum of 5 to 10 mm of Hg. The disposable columns were conditioned by passing 2 ml of methanol through the column followed by two (2 ml) rinses with double glass-distilled water at a vacuum of 380 mm of Hg.

The eluate was placed into crimp-top vials for analysis by gas chromatography. The Hewlett-Packard 5880A gas chromatography unit contained a 15 m, 0.24 mm I.D. fused silica column with a liquid-film thickness of 1.0 μm (J & W Scientific, Inc., Rancho Cordova, CA). A flame ionization detector was used with 3 μl splitless injections. Temperature program was 30°C per min from 70°C to 220°C and 15°C per min to 320°C.

Daily estimates of the accuracy of the analytical methodology were obtained by standard additions of BBP to both control chamber water (with fish) and headtank water (without fish). Both types of seawater were spiked to evaluate the effect of fish slime on the extraction procedure. Daily samples were taken from each chamber, and estimates of the analytical precision were obtained from triplicate samples from the lowest BBP treatments on at least two different days in each bioassay. BBP (98% purity) for standardization and dosing was obtained from Pflatz and Bauer, Stamford, CN, catalog #B27750.

Analysis of Catecholamines

At the end of a 96-hr bioassay, surviving fish were placed into plastic bags and immediately immersed in dry-ice cooled acetone until frozen. After three weeks of storage at −25°C, the brains were excised, weighed, homogenized, and the catechloamines were extracted from individual whole brains by the method of Maruyama *et al.* (1980). Quantification was by high performance liquid chromatography with electrochemical detection using the method of Smith and Sleet (1982). Twenty μL of extract was

[1] Mention of trade names or commercial products does not constitute endorsement or recommendation for use

injected into a C-18 precolumn (LiChrosorb® manufactured by MC/B Manufacturing Chemists, Inc., Cincinnati, OH) and separated with an Ultrasphere® C-18 column (Beckman Instruments, Inc., Redmond, WA). The mobile phase was 0.04 M sodium citrate (pH 5.0) and the electrode was set at +0.6 volts.

Determination of LC_{50}

The LC_{50} values were based on the average, daily-measured concentration in each treatment rather than the nominal concentration. Logistic regression of average concentrations and mortalities, using the method of maximum likelihood, was used to estimate the LC_{50}s with confidence intervals generated using a heterogeneity factor (Finney 1971). When mortalities were present in only one treatment, LC_{50} values were estimated without confidence intervals from straight-line graphical interpretation (APHA 1976).

Results

Acute Toxicity

One 8-day bioassay (A) and two simultaneous 96-hr bioassays (B and C) were conducted in September and October of 1981. The 96-hr LC_{50}s with 95% confidence limits for bioassays B and C were 0.51 mg/L (0.47 mg/L to 0.56 mg/L) and 0.51 mg/L (0.46 mg/L to 0.55 mg/L), respectively. Confidence intervals were incalculable at 96 hr for bioassay A because mortalities before 105 hr had occurred at only one treatment level. After 105 hr, the time course of toxicity (Figure 1) approached an asymptotic LC_{50} of 0.49 mg/L (0.45 mg/L to 0.56 mg/L) at 165 hr. The average of the daily-measured concentrations was 76% of the nominal exposure concentrations (Table 1).

Sublethal Effects

Behavioral effects were observed at concentrations below the LC_{50} in the three bioassays (Table 2). After 3 hr of exposure, the minimum school density (school occupying entire chamber) was seen at concentrations as low as 0.27 mg/L with coughing and a reduction in activity at all higher concentrations. After 96 hr of exposure, the minimum school density was seen in all treatments greater than 0.2 mg/L. At 0.16 mg/L the school density was less than at 0.08 mg/L which was less than the control. Fish with dorsal darkening were seen at concentrations at and above 0.48 mg/L within 24 hr of exposure. As time progressed, fish at lower treatments darkened so that by 96 hr all fish at and above 0.30 mg/L, and a few fish at 0.24 mg/L, were darker than the controls. In general, those fish exposed to lethal

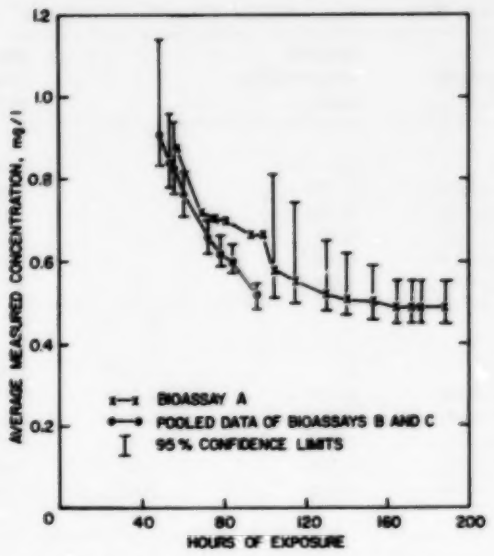

Fig. 1. LC_{50} values for shiner perch as a function of the time of exposure to butylbenzyl phthalate

concentrations scattered throughout the tanks, quickly darkened, experienced a gradual loss of equilibrium with paralysis (tail bent), and floated head down at the surface before settling to the bottom where death occurred. While equilibrium was being lost, their coughing rate increased to 1–3 per second followed by opercular flairing with mouth open.

Analysis of Butylbenzyl Phthalate

There was no significant difference between the percent recoveries of BBP additions in bioassay A and B, C, and the presence or absence of fish did not significantly affect the recovery of BBP during either bioassay. The mean recovery of BBP was 97% ± 9.9%, n = 23. The mean coefficient of variation for triplicate samples from the treatments was 4%, n = 7, for a range of from 0.06 to 0.25 mg/L.

Brain Catecholamines

The levels of epinephrine (EP) and norepinephrine (NE) in the whole brains of fish from experiment B, C were approximately one order of magnitude higher than the dopamine (DA) levels (Figure 2). The only significant treatment effect was a decrease in EP levels in fish exposed to 0.42 mg/L BBP for 96 hr.

Table 1. Exposure concentrations of butylbenzyl phthalate

Experiment (date)	Nominal concentrations mg/L	Measured concentrations (from daily samples) mg/L			% of Nominal
		$\bar{x} \pm s$			
A	0	0.002 ± 0.0007, n = 5			—
9/21–29/81	0.130	0.080 ± 0.0168, n = 4			62
9/21–29/81	0.216	0.155 ± 0.0258, n = 4			72
9/21–29/81	0.360	0.27 ± 0.031, n = 4			75
9/21–29/81	0.600	0.48 ± 0.076, n = 4			80
9/21–29/81	1.000	0.92 ± 0.199, n = 4			92
		B + C	B	C	
		$\bar{x}$	$\bar{x} \pm s$,	$\bar{x} \pm s$	
B,C	0	0.0023	0.002 ± 0.0007,	0.002 ± 0.0004, n = 4	—
10/6–10/81	0.316	0.236	0.226 ± 0.0142,	0.246 ± 0.0237, n = 5	74,78
10/6–10/81	0.422	0.30	0.296 ± 0.0228,	0.30 ± 0.030, n = 5	70,71
10/6–10/81	0.563	0.42	0.45 ± 0.042,	0.393 ± 0.023, n = 5	80,70
10/6–10/81	0.750	0.57	0.58 ± 0.080,	0.55 ± 0.032, n = 5	77,73
10/6–10/81	1.000	0.82	0.82 ± 0.151,	0.83 ± 0.140, n = 5	82,83
					$\bar{x}$ = 76

Table 2. Sublethal effects observed after 96 hr of exposure to butylbenzyl phthalate

Concentration BBP, mg/L	Activity decrease	Coughing presence	Coloration dorsal darkening	School decreased density
0.4–1.0	XX	XX	XX	+ + +
0.2–0.4	X	X	X	+ + +
0.16	—	—	—	+ +
0.08	—	—	—	+

Percent of fish affected: 100% (XX), at least 50% (X), 0% (—). Area of chambers occupied by fish school: 100% (+ + +), 70% to 90% (+ +), 50% to 70% (+), control chambers: 30% to 50% occupied

Discussion

Acute Toxicity

There is a paucity of BBP toxicity data for marine fish. LC_{50} values from the same laboratory range from 3 mg/L (Gledhill *et al.* 1980) to 440 mg/L (Heitmuller *et al.* 1981) for sheepshead minnow (*Cyprinodon variegatus*). These values were determined in static bioassays using nominal concentrations. The dosing method (direct addition of the compound, or of a solution) was not specified in either report. Several factors which may have contributed to the higher of these two values include incomplete solution, rapid surface absorption (Sullivan *et al.* 1981), and biodegradation of the phthalate ester (Taylor *et al.* 1981).

Additional work in our laboratory using both a replacement static bioassay procedure and the same flow-through bioassay scheme as this study, Randall *et al.* (in press) found LC_{50} values based on measured concentrations of 0.66 mg/L and 0.58 mg/L, respectively, for juvenile English sole (*Parophrys vertulus*). They also reported that the concentration of BBP decreased rapidly in static seawater bioassay chambers with a rate constant of approximately 0.5 per day.

The EPA, in recommending water quality criteria for phthalate esters (USEPA 1980b), reported that the Heitmuller *et al.* (1981) value resulted from the addition of the pure compound, while a solution dosing scheme was used in Gledhill *et al.* (1980). Considering that the 2.9 mg/L solubility of BBP in deionized water (Gledhill *et al.* 1980) would be lower in seawater, due to a salting-out effect, and that BBP can rapidly disappear from static seawater bioassay chambers, the 440 mg/L LC_{50} of Heitmuller *et al.* should be discounted.

Interspecies differences in the susceptibility to this toxicant could account for some of the differences between LC_{50} values reported for sheepshead minnow and those found for English sole and shiner perch. However, systematic differences in the way

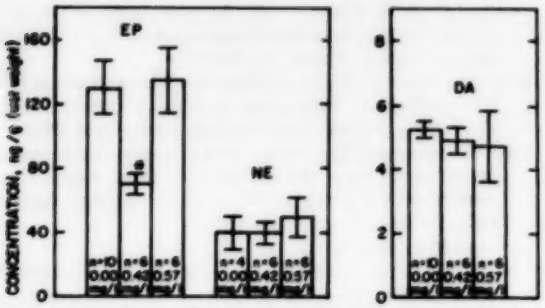

Fig. 2. The effects of 96 hr exposure to butylbenzyl phthalate on the catecholamine levels in the whole brain of shiner perch (*Cymatogaster aggregata*)

*** Significantly different from the control fish (<0.05)**

I, Indicates the 95% confidence interval

EP, epinephrine; NE, norepinephrine, DA, dopamine (note smaller scale)

the bioassays were conducted suggest that the actual exposure levels in the sheepshead minnow studies were much lower than the nominal concentrations which were used to calculate the LC_{50}s. Taking this into account, there would be much less difference between the LC_{50} values reported for these three species of marine fish.

The results of standardized bioassays with individual organic compunds are often used in product licensing and establishing water quality standards. These results should be as accurate as possible. The relative insolubility, high surface adsorption, and potential degradation of many organic compounds make LC_{50} values which are based on nominal concentrations potentially inaccurate as well as environmentally meaningless, *e.g.*, a published LC_{50} exceeding the solubility of BBP by a factor of >100. Therefore, toxicity studies of individual organic compounds using nominal concentrations should be discouraged.

Behavioral Responses and Cathecholamines

The behavioral changes observed in shiner perch suggest that BBP affects the central nervous system (CNS) because of the significant involvement of the forebrain in schooling behavior (Aronson and Kaplan 1968) and the neural control of color in teleosts (Healey and Ross 1966). The sluggish behavior observed in this study has also been observed in striped mullet exposed to fluorene analogs (Thomas *et al.* 1981), whereas increased motor activity was observed in Gulf killifish exposed to Aroclor 1242® (Fingerman and Russell 1980). However, in both of these studies, reduced levels of brain catecholamines were found.

No consistent dose-dependent decrease in epinephrine was found in this study. The significant decrease seen in the 0.42 mg/L treatment was not found at 0.57 mg/L (Figure 2). At the higher exposure, only 11 close-to-death fish remained at 96 hr whereas at 0.42 mg/L, 35 fish were still alive. This suggests that the effect BBP may have on brain levels of EP is counteracted by processes which occur as the fish approaches death.

The limited scope of our neurochemical measurements does not permit the presentation of a possible mechanism for the action of BBP on the CNS. These measurements and observations do suggest that the mode of toxic action for BBP is through its influence on the central adrenergic nervous system. In addition, at concentrations as low as 0.08 mg/L, behavior which can be essential to survival in the wild can be altered.

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